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Immunology

Cytolytic T-cell clones against an autologous human melanoma: Specificity study and definition of three antigens by immunoselection

ALEXANDER KNUTH*†, THOMAS WÖLFEL*, EVA KLEHMANN*, THIERRY BOON‡, AND KARL-HERMANN MEYER ZUM BÜSCHENFELDE*

*I. Medizinische Klinik und Poliklinik, Johannes Gutenberg-Universität, Langenbeckstrasse 1, D-6500 Mainz, Federal Republic of Germany; and ‡Ludwig Institute for Cancer Research, Brussels Branch, 74 avenue Hippocrate, UCL 74.59, B-1200 Brussels, Belgium

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ABSTRACT **Cytolytic T-lymphocyte (CTL) clones against an autologous melanoma (SK-MEL-29) were generated by mixed lymphocyte tumor culture and subsequent cloning of responder lymphocytes at limiting dilutions. These CTL clones lysed autologous melanoma but not autologous Epstein–Barr virus-transformed B cells and none of the allogeneic tumor targets included in the specificity analysis. The lysis of autologous melanoma targets could be inhibited by monoclonal antibodies against monomorphic HLA class I determinants. For proliferation of CTLs, the stimulation with the relevant target antigen on autologous tumor cells was essential. Immunoselection experiments carried out with two CTL clones revealed the existence of melanoma subclones that were resistant to lysis by the CTL clones used for immunoselection but were still lysed by other autologous CTL clones. This analysis allowed us to identify three stable simultaneously expressed antigens on the melanoma cells defined by autologous CTLs.**

The search for human cancer antigens eliciting a specific autologous immune response has been pursued with serum antibodies and T lymphocytes. The most compelling evidence to date for the existence of tumor antigens eliciting a specific autologous antibody response in patients comes from studies of Old and colleagues (1–3), demonstrating serum autoantibodies that were specific for the autologous cancer cells. Several groups obtained cytolytic T-lymphocyte (CTL) clones that showed specificity for the autologous tumor cells (4–8). We have developed techniques for the systematic production of stable CTL clones directed against autologous melanoma cells (9). In a study of a melanoma patient, MZ-2, three antigens were identified with autologous CTL clones: antigen A was present on all melanoma clones tested, antigen B could be lost during long-term culture, and antigen C was only expressed on a minority of melanoma clones (9).

We report here a specificity study of autologous CTL clones obtained against a melanoma SK-MEL-29 from a patient AV. Through immunoselection with CTL clones we were able to define three stable antigens on this melanoma.

MATERIALS AND METHODS

Patient. Patient AV is a 35-year-old Caucasian male. In 1976 and 1978 he underwent resections of extensive metastatic melanoma of axillary, supraclavicular, and cervical lymph nodes. Since that time he has remained free of detectable melanoma. Between 1976 and 1978, the patient's lymphocytes were found to be strongly cytotoxic for autologous cultured melanoma cells while there was still evidence of residual disease (10). Later, when the patient presented with no evidence of residual disease, only minimal peripheral lymphocyte cytotoxicity was detected for autologous melanoma cells in culture. No serum autoantibodies reactive with cell surface antigens of autologous melanoma cells were found in the patient's serum at any time.

Cell Lines. If not indicated otherwise, cell lines used in this study were a generous gift of L. J. Old (Memorial Sloan-Kettering Cancer Center, New York) and have been described elsewhere (2, 3, 5, 11–13). SK-MEL-29 was cloned by limiting dilution, and five clones (SK-MEL-29.1 to -5) were selected for further experiments. The stomach cancer cell line MZ-Sto-1, the hepatoma cell line MZ-Hep-1, and the cell lines of extrahepatic biliary tract cancer, MZ-ChA-1 and SK-ChA-1, have been described (14–16). AK-EBV is an Epstein–Barr virus (EBV)-transformed lymphoblastoid cell line established from a healthy donor.

Tumor cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 10 mM Hepes buffer, L-arginine (116 mg/ml), L-asparagine (36 mg/ml), L-glutamine (216 mg/ml), penicillin (100 international units/ml), streptomycin (100 μg/ml), and 10% (vol/vol) fetal calf serum (FCS). This medium is hereafter referred to as M^a. EBV-transformed B-cell (EBV-B) lines were cultured in RPMI 1640 medium (M^b), supplemented as described for M^a. All cultured cells were kept in a water-saturated atmosphere with 5% CO_2/95% air at 37°C.

Mixed Lymphocyte Tumor Cell Culture (MLTC). Peripheral blood mononuclear leukocytes were separated from heparinized blood in 1980 and 1982 by centrifugation on a Ficoll-Paque cushion (Pharmacia) and kept in frozen storage in aliquots of 5–10 × 10^6 in 90% FCS and 10% dimethyl sulfoxide in the vapor phase of a liquid nitrogen bank. MLTC was performed as described (9). Briefly, 10^6 peripheral blood mononuclear leukocytes were cocultured with irradiated (100 Gy, ^{137}Cs source) bulk culture (SK-MEL-29) or clones of autologous melanoma (SK-MEL-29.1, -3, and -4) at 10^5 cells in 2 ml of RPMI 1640 medium supplemented as described above, but with 10% (vol/vol) human serum (medium M^c). Human recombinant interleukin 2 [IL-2, a generous gift of Biogen (17)] was added from day 3 on at a final concentration of 10 ng/ml. After 1 week, 3 × 10^5 responder cells were restimulated with 5 × 10^4 irradiated stimulator cells at weekly intervals in 2 ml of M^c.

Derivation of CTL Clones. Production of stable CTL clones has been described in detail (9). Briefly, after 7 days in culture, MLTC responder lymphocytes were seeded at 1.0 cell per well in round-bottom 96-well microtiter plates (Greiner, Nürtingen, F.R.G.) in 200 μl of culture medium M^c supplemented with IL-2 (IL-2 M^c) as described above that

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Abbreviations: CTL, cytolytic T lymphocyte; EBV, Epstein–Barr virus; FCS, fetal calf serum; IL-2, human recombinant interleukin 2; MLTC, mixed lymphocyte tumor cell culture.

†To whom reprint requests should be addressed.

had been seeded with autologous melanoma cells as stimulators (3×10^3 cells per well) and AK-EBV as feeder cells at 10^4 cells per well. Feeders and stimulators were irradiated as described above.

After 7 days in culture 100 μl of supernatant was removed from each well and replaced by 100 μl of IL-2 M^c with fresh stimulator and feeder cells. Lymphocyte colony growth was checked at regular intervals from day 12 on, and fast growing colonies were transferred to 24-well Linbro Plates (Nunc) containing 5×10^4 irradiated stimulator cells and 2×10^5 irradiated feeder cells per well in 2 ml of IL-2 M^c. Slower growing colonies were first transferred for ≈1 week to flat bottom 96-well microtiter plates (200 μl per well) (Greiner).

On day 21 colonies were tested for lytic activity in a small scale screening assay against autologous melanoma cells and K562 target cells to eliminate cultures with natural killer activity. Selected CTL clones were restimulated at 4- to 7-day intervals at $1–3 \times 10^5$ CTL per well in Linbro wells under the conditions described.

Assay for Cytolytic Activity. Target cells were incubated at 10^7 cells per ml in FCS for 45 min at 37°C with $Na(^{51}Cr)O_4$ (Institut des Radioelements, Fleurus, Belgium) at 200 μCi/ml (1 Ci = 37 GBq), washed three times, and resuspended at 10^4 cells per ml of medium M^a. Effector lymphocytes were serially diluted in 100 μl of medium M^a in conical 96-well microtiter plates (Greiner). After adding 100 μl of labeled target cell suspension plates were incubated for 4 hr at 37°C in a 5% CO_2/95% air atmosphere. The plates were centrifuged at $200 \times g$ for 5 min, 100 μl of supernatant was collected, and radioactivity was measured in a γ counter (Kontron, Zurich). The percentage of specific ^{51}Cr release was calculated with the formula: % specific chromium release = (experimental ^{51}Cr release − spontaneous ^{51}Cr release) × 100/(maximum ^{51}Cr release − spontaneous ^{51}Cr release). Maximum ^{51}Cr release was obtained by adding 100 μl of Triton X-100 (Sigma) to labeled target cells. Spontaneous ^{51}Cr release ranged between 5 and 25% of total label incorporated into cells.

MLTC-derived responder colonies were initially screened in a microcytotoxicity assay. Lymphocyte cultures in microtiter plates (200 μl per well) were split in 60-μl aliquots after resuspending lymphocytes and added to labeled target cells in microtiter test plates to a final volume of 200 μl. These samples were processed as described above.

Stimulation Assays. Proliferation of CTLs to specific antigen stimulation with irradiated tumor cells was tested in round-bottom 96-well microtiter plates (Greiner) in 200 μl of medium M^c per well. Stimulating tumor cells were seeded at 2×10^3 cells per well, feeder lymphoblastoid cells were at 2×10^4 cells per well, and both were irradiated with 100 Gy. CTLs were seeded at 7×10^2 or 2×10^3 cells per well. After 5 days, microcultures were pulse-labeled with 1.0 μCi of [^{3}H]thymidine (specific activity, 2.0 Ci/mmol; NEN), and harvested after 6 or 12 hr with a suction PHD cell harvester (Cambridge Technology, Cambridge, MA) onto glass fiber strips. Incorporated radioactivity was determined as a mean of quadruplicates in a liquid scintillation counter (LS 1801, Beckman).

Serological Reagents. Monoclonal antibodies used were as follows: W6/32 against monomorphic HLA-class I determinants (18); HB55 against monomorphic HLA-class II determinants (19); and OKT8, OKT3, and 12T4D11 antibodies against CD8, CD3, and CD4 determinants on T lymphocytes, respectively (a generous gift of S. C. Meuer, Heidelberg). NS-1 ascites fluid was used as a negative control. All antibodies were used in ascites fluid with an approximate protein concentration of 15–20 mg of protein per ml.

Flow Cytometric Analysis. Cells were incubated with murine monoclonal antibodies at saturating concentrations for 30 min on ice, washed, and stained with polyclonal goat anti-mouse $F(ab')_2$ antibody fragments coupled with fluorescein isothiocyanate (Coulter) for another 30 min on ice. Specimens were analyzed in an Epics V cell sorter (Coulter).

Inhibition of Cytolysis with Monoclonal Antibodies. Duplicate samples of ^{51}Cr-labeled target cells (10^3 cells per well) were preincubated with serial dilutions of antibodies in medium M^a in conical 96-well microtiter plates (Greiner) at 4°C for 30 min. Then effector cells (3×10^4 cells per well) were added to give a final volume of 200 μl per well. After a 4-hr incubation period at 37°C in a 5% CO_2/95% air atmosphere, plates were harvested and processed as described for cytotoxicity assays. Controls always included incubation of antibodies with targets alone to exclude antibody-dependent mechanisms of cell lysis in the absence of CTLs. Later experiments showed that preincubation of target cells with monoclonal antibodies was not required for efficient inhibition of CTL lysis.

Immunoselection with CTL Clones. SK-MEL-29 cultures were cloned at limiting dilutions and clone SK-MEL-29.1 was selected for further studies. Immunoselection experiments were designed as described by Maryanski and Boon (20). The target cell line SK-MEL-29.1 was mutagenized by irradiation with 10 Gy (^{137}Cs source). AV-CTL clones 2/9 and 5/57 were mixed in 12-fold (CTL 2/9) or 16-fold (CTL 5/57) excess with 3×10^6 target cells in aliquots of 5×10^5 cells each in 5 ml of medium M^a in 10-ml round bottom tubes (Greiner) and centrifuged at $150 \times g$ for 5 min. After a 6-hr incubation at 37°C, cells were washed and resuspended in medium M^a with a supplement of 20% FCS. The remaining cell suspension was split in half. One part was transferred to a T60 tissue culture flask (Greiner) in a final volume of 20 ml of medium M^a with 20% FCS as a source of cells for a second selection, if necessary. The other half of selected cells was plated at limiting dilutions in flat bottom 96-well microtiter plates (Greiner) starting at 6×10^3 cells per well. Each well contained 200 μl of medium M^a with 20% FCS. To improve chances of recovery for single immunoselected melanoma clones, NIH 3T3 cells were irradiated with 100 Gy and added as feeder cells to T60 culture flasks and wells of microtiter plates (10^4 cells per well) (21).

DNA Extraction and Southern Blot Analysis. High molecular weight genomic DNA was prepared from tumor cells or CTL as described (22). CTLs were separated from cell debris on a Ficoll-Paque cushion (Pharmacia) before DNA extraction. The molecular weight of the extracted DNA was higher than that of phage λ, as demonstrated by agarose gel electrophoresis.

Southern blot hybridization with a T-cell receptor β gene probe (a generous gift of Tak Mak, Ontario Cancer Institute, Toronto) was performed as described (22).

RESULTS

Derivation and Characterization of CTL Clones Directed Against Autologous Melanoma. MLTCs were established with peripheral blood mononuclear leukocytes and the melanoma line SK-MEL-29 derived from patient AV. After 7 days, the responder lymphocytes were cloned by limiting dilutions. The cloned lymphocytes were restimulated with SK-MEL-29 and their lytic activity was tested against SK-MEL-29 and natural killer target K562. Of 3500 wells seeded at one responder cell per well, 146 proliferating clones were obtained. Forty-five clones showed lytic activity and 34 clones lysed SK-MEL-29 but not K562. From these, a number of fast growing clones with a stable lytic activity were chosen. Long-term culture of these clones required restimulation with irradiated autologous melanoma cells in the presence of allogeneic AK-EBV-B cells and IL-2 as shown in Fig. 1.

In immunofluorescence studies by flow cytometry all tested AV-CTL clones (2/9, 3/7, 3/9, 5/57, and 5/76)

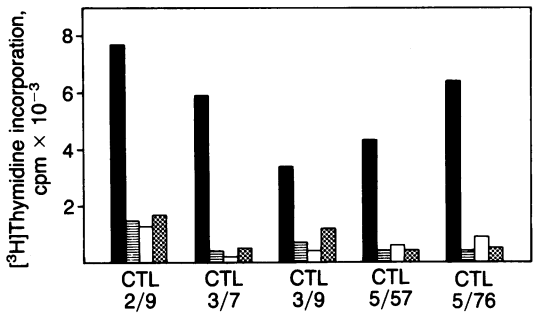

FIG. 1. Conditions of CTL proliferation. The *in vitro* proliferation of AV-CTL clones (initially seeded at 2000 CTLs per well) was measured by [^{3}H]thymidine incorporation in the presence of autologous melanoma, feeder cells, and recombinant IL-2 (■); autologous melanoma and recombinant IL-2 (▣); feeder cells and recombinant IL-2 (□); and autologous melanoma and feeder cells (▨).

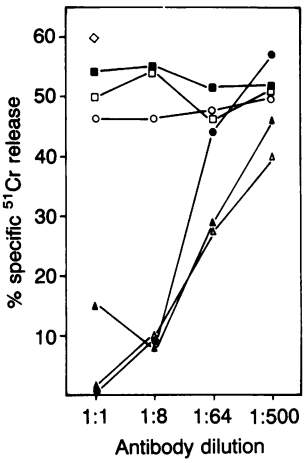

FIG. 2. CTL inhibition with antibodies. Lysis of SK-MEL-29.1 target cells by AV-CTL clone 5/57 at an effector cell/target cell ratio of 30:1 was inhibited in a 4-hr ^{51}Cr release assay by the following antibodies: W6/32 (monomorphic HLA-class I determinants) (●), Okt8 (CD8) (▲), and Okt3 (CD3) (△). ^{51}Cr release was not inhibited by antibodies HB55 (monomorphic HLA-class II determinants) (○) and 12 T4 D11 (CD4) (■) or by NS-1 (mouse ascites) (□). Lysis in the absence of antibody is also shown (◇).

displayed the phenotype T3$^+$, T4$^-$, T8$^+$, HLA class I$^+$, HLA class II$^+$. A panel of monoclonal antibodies was tested for the inhibition of specific AV-CTL activity against autologous AV melanoma target cells. Monoclonal antibody W6/32, directed against a monomorphic determinant of HLA-class I molecules, and antibodies OKT3 and OKT8, directed against CD3 and CD8 differentiation antigens on human T lymphocytes, inhibited the lysis of autologous melanoma targets by all AV-CTL clones tested as shown for CTL clone 5/57 in Fig. 2. Monoclonal antibodies recognizing CD4 or monomorphic determinants on HLA-class II antigens did not inhibit the specific activity of AV-CTL clones against autologous tumor target cells. T-cell receptor β genes were rearranged in all

Table 1. Specificity analysis of AV-CTL activity

		% specific ^{51}Cr release														
		CTL 2/9			CTL 3/7			CTL 3/9			CTL 5/57			CTL 5/76		
Target	Line	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
Autologous																
Melanoma	SK-MEL-29*	56	60	52	52	28	9	61	61	43	60	55	44	61	53	43
	SK-MEL-29.1				46	22	8				74	66	58	60	59	41
	SK-MEL-29.2				51	30	11	50	52	40	61	52	41	55	46	29
	SK-MEL-29.3				60	36	21	55	52	48	62	62	55	66	62	42
	SK-MEL-29.4				47	30	13	45	45	38	70	54	29	76	71	51
	SK-MEL-29.5				46	25	8	57	54	40	45	45	37	43	41	32
Lymphoblast	AV-EBV	3	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Allogeneic																
Melanoma	MZ-MEL-2*				4	0	0	6	7	6	14	7	1	14	7	0
	SK-MEL-37*				0	0	0	0	0	0	5	2	0	4	1	1
	SK-MEL-40*				5	4	6	10	4	6	4	0	0	4	0	0
	SK-MEL-64*				1	0	0	0	1	0	0	0	0	0	0	0
	SK-MEL-97*				2	1	2	3	3	2	6	2	0	1	2	0
	SK-MEL-88*				2	1	0	3	1	2						
	SK-MEL-13*				2	2	1	4	3	0						
	SK-MEL-31*				1	0	0									
	SK-MEL-152				4	3	0									
	SK-MEL-19*										8	5	0	7	1	0
	SK-MEL-63*										2	0	0	0	0	0
Renal cancer	SK-RC-7	10	8	9	14	10	9	17	13	8	12	9	9	14	11	11
	SK-RC-11	0	0	1	1	0	0	6	2	1	2	0	0	4	2	0
	SK-RC-18	0	0	0	0	0	0	5	3	0	0	1	0	1	0	0
	SK-RC-6	2	0	0	4	1	0	3	3	0	4	3	1	3	2	2
Lung cancer	SK-LC-1				0	2	0	0	0	0	1	0	0	0	0	0
	SK-LC-2	4	1	1	5	2	3	0	7	3	0	2	0	4	2	2
Breast cancer	MCF-7	4	4	4	3	3	6	22	15	12	0	1	2	9	5	1
Bladder cancer	RT-4	0	5	2	0	0	3	5	1	3	2	2	0	5	3	3
Gastric cancer	MZ-Sto-1										2	1	0	1	0	0
Pancreatic cancer	Capan 2	0	3	2	0	0	3	2	1	4	0	0	0	0	0	0
Cervix cancer	ME-180	0	2	2	6	3	1	12	8	3	2	2	0	2	0	3
Hepatoma	Mz-Hep-1										4	3	0	8	3	1
Glioblastoma	AN	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Neuroblastoma	SK-NMC				0	0	0	3	1	1						
Leukemia	K562	1	0	0	1	0	1	2	5	1	8	2	1	7	6	1

AV-CTL clones were cultured at effector cell/target cell ratios of 10:1 (columns a), 3:1 (columns b), or 1:1 (columns c).

*HLA class I allospecificities shared with SK-MEL-29 phenotype (*A2,28; B12,w4,w6; Cw5,w6*): *A2* (SK-MEL-19, SK-MEL-31, SK-MEL-37, SK-MEL-40, SK-MEL-63); *A28* (SK-MEL-13); *Bw4* (MZ-MEL-2, SK-MEL-63, SK-MEL-64, SK-MEL-88, SK-MEL-97); *Bw6* (SK-MEL-40, SK-MEL-63, SK-MEL-88, SK-MEL-97); *Cw4* (SK-MEL-88); *Cw6* (MZ-MEL-2, SK-MEL-40, SK-MEL-63, SK-MEL-64).

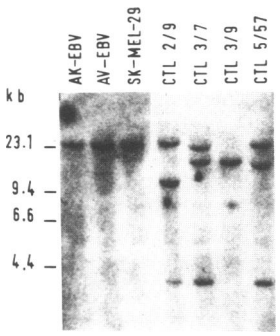

FIG. 3. Southern blot analysis of DNA from AV-CTL clones with a T-cell receptor gene probe. CTL DNA (20 μg per lane) was digested with *Bam*HI, size-fractionated by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized with a ^{32}P-labeled cDNA probe that contains constant-region and joining-region segments of a T-cell receptor β-chain gene (23, 24). *Hin*dIII fragments of λ phage DNA were used as size markers. kb, Kilobases.

AV-CTL clones examined. The observed patterns were clearly different from each other (Fig. 3).

Specificity of CTL Clones. Five CTL clones were tested on several SK-MEL-29 clones. All melanoma clones were lysed equally. No significant lysis of an autologous EBV-transformed lymphoblastoid cell line was observed (Table 1). The specificity of lytic activity of AV-CTL as tested against autologous AV melanoma cultures, autologous AV-EBV-B cells and K562 target cells was stable at least for a period of 4 weeks. With AV-CTL clone 3/7, we followed this stability of specific cytolytic activity for 169 days, as demonstrated in Fig. 4.

The CTL clones were also tested against a panel of allogeneic tumor cells of various histological types (Table 1). The lytic activity observed on these tumors was always lower than 5% of that observed on the autologous melanoma cells except for renal cancer SK-RC-7 and breast cancer MCF-7. In proliferation assays, a number of allogeneic tumors were incapable of acting as stimulator cells for CTL clone 5/76 (Fig. 5). SK-RC-7 and MCF-7 did not induce any stimulation. HLA class I antigens of allogeneic melanoma lines shared with SK-MEL-29 are listed in Table 1.

Immunoselection Experiments with CTL Clones. CTL clones, 2/9 and 5/57, derived from independent MLTC experiments were used for immunoselection studies. The melanoma clone SK-MEL-29.1 was irradiated with 10 Gy to increase the probability of genetic lesions that might result in the loss of expression of antigens recognized by these CTLs. For immunoselection experiments, 3×10^6 SK-MEL-29.1 cells were incubated with an excess of CTL clone 2/9 or 5/57, as described above.

After immunoselection with CTL clone 2/9, four surviving melanoma clones were found on limiting dilution plates out of 1.8×10^5 cells seeded initially at 3×10^3 cells per well. Only one of these four clones, SK-MEL-29.1.109, was resistant to the lysis by the selecting CTL clone 2/9 in a 4-hr ^{51}Cr-release assay. No survivors were found at higher cell concentrations. Six surviving tumor cell clones were found after immunoselection with CTL clone 5/57 out of 6×10^5 initial cells plated at 6×10^3 cells per well. One of them, SK-MEL-29.1.14, was resistant to CTL clone 5/57. The immunoselected antigen-loss variants remained resistant to the selecting CTL clones for at least 4 months of continuous culture. Both variants were susceptible to lysis by the other nonimmunoselecting CTL clones to which we refer in this study (Fig. 6; data for CTL clones 3/7 and 3/9 not shown). In addition, the variants had lost the growth-stimulating ability for the selecting CTL clone (Fig. 7).

By derivation of antigen-loss variants, we defined two stable antigens simultaneously expressed on SK-MEL-29 clones. Antigen A was recognized by CTL clone 2/9, and

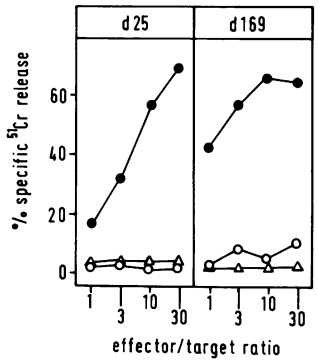

FIG. 4. Stable lytic activity of CTL clone 3/7 over time. The lytic activity was tested in ^{51}Cr release assays on day 25 (d25) and day 169 (d169) against autologous tumor cells SK-MEL-29.1 (●), autologous AV-EBV lymphoblastoid cells (○), and K562 (△).

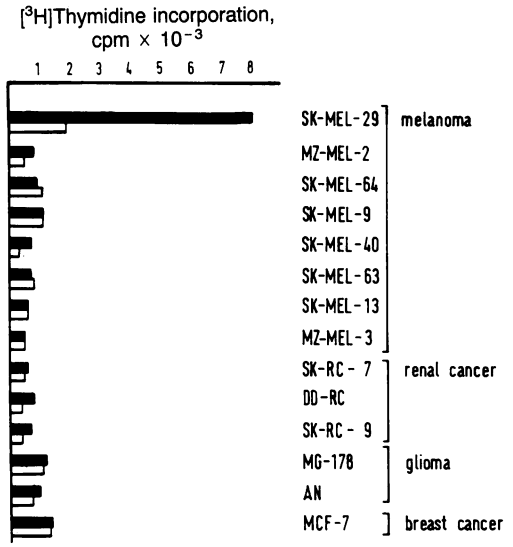

FIG. 5. Specificity analysis of proliferative response of AV-CTL clone 5/76. Allogeneic tumor lines as compared with the autologous melanoma line SK-MEL-29 were tested for their ability to stimulate CTL proliferation, as measured by [^{3}H]thymidine incorporation of lymphocytes (initially seeded at 2000 CTLs per well) and irradiated stimulator and feeder cells (solid bars) and [^{3}H]thymidine incorporation of irradiated stimulator and feeder cells alone (open bars).

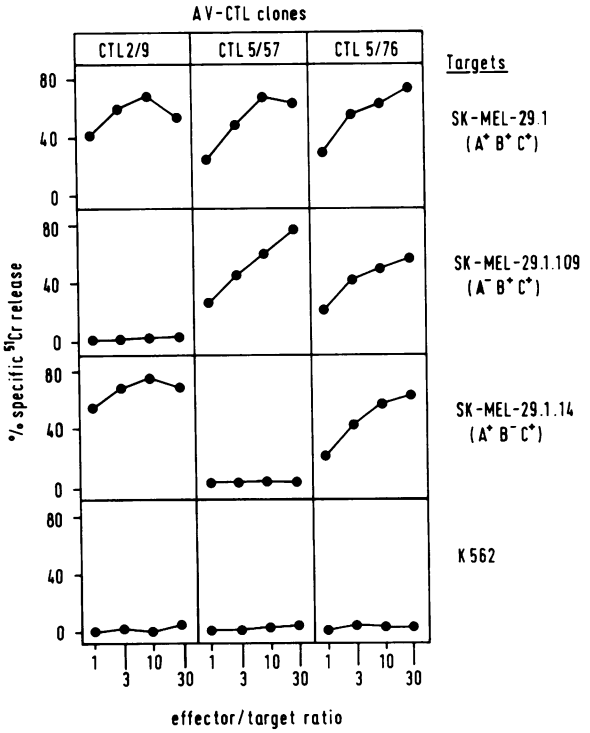

FIG. 6. Cytolytic activity of AV-CTL clones against immunoselected tumor cell variants. SK-MEL-29.1.109, immunoselected with CTL clone 2/9, and SK-MEL-29.1.14, immunoselected with CTL clone 5/57, were derived from clone SK-MEL-29.1 and had lost antigen A or B, respectively.

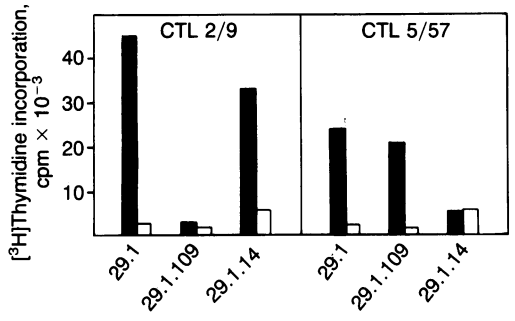

FIG. 7. Proliferative response of AV-CTL clones 2/9 and 5/57 to immunoselected variants SK-MEL-29.1.109 and SK-MEL-29.1.14 as compared with SK-MEL-29.1. [^{3}H]Thymidine incorporation of lymphocytes (initially seeded at 700 CTLs per well) and irradiated stimulator and feeder cells (solid bars) and [^{3}H]thymidine incorporation of irradiated stimulator and feeder cells alone (open bars) are shown.

antigen B was recognized by CTL clone 5/57. The existence of at least one other stable antigen is assumed since three other CTL clones can lyse both antigen-loss variants.

DISCUSSION

CTL clones were derived from peripheral blood of a patient AV several years after the first clinical manifestation of metastatic melanoma, when the patient no longer had clinical evidence of disease. Stimulation of lymphocytes in MLTC with autologous AV melanoma cells *in vitro* and subsequent cloning of MLTC responders rendered stable CTL clones.

In a specificity analysis of various CTL clones, we found specificity of cytolysis and proliferation induction for the autologous melanoma cells. By means of direct cytotoxicity testing, no differences in antigen expression of autologous melanoma clones were detected, as opposed to another melanoma system (9). There are two possible explanations for this observation: either all CTL clones recognize the same antigen or different antigens are simultaneously expressed on the melanoma cells.

The simultaneous expression of multiple stable T-cell-defined antigens on tumor cells was demonstrated in several mouse tumor models *in vivo* and *in vitro* by using monoclonal T-cell probes as immunoselectants. In mouse mastocytoma P815, single-step immunoselection with CTL clones uncovered stable antigen-loss variants in chemically mutagenized tumor variants expressing tumor rejection antigens (tum$^-$) (20). These *in vitro* studies correlated with *in vivo* findings in mice where mutagen induced tumor rejection antigens were found to be lost *in vivo*, allowing mastocytoma cells to escape from CTL control (25). Others described antigen-loss variants and the simultaneous expression of multiple tumor antigens in UV-induced immunogenic murine fibrosarcoma after selection with monoclonal T-cell probes *in vitro* (26).

In human tumors, clonal heterogeneity with respect to quantitative expression of melanoma antigens, as detected with autologous serum antibodies (13) as well as with CTL probes (27), has been shown. On the other hand, qualitative differences of melanoma clones in expression of unstable CTL-defined antigens has also been described (9). Here we demonstrate the simultaneous expression of three stable CTL-defined antigens on AV melanoma cells by *in vitro* immunoselection (Fig. 6). The extent to which antigenic diversity may be unmasked by further immunoselection experiments is unclear, yet. We have isolated CTL clones specific for antigens A and B (data not shown) from peripheral blood mononuclear leukocytes of patient AV, obtained in 1987—i.e., 9 years after the last clinical evidence of melanoma. Apparently, precursor lymphocytes recognizing these two antigens persist in the patient's blood.

In view of cancer progression and metastasis, it may be speculated that antigen loss variants may escape the specific control of immunorecognition by CTLs, giving rise to further spread of tumor. It is, therefore, important to establish whether human tumors often carry several different tumor-associated antigens, since this should decrease their ability to escape the immune defense by antigen loss.

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